ORIGINAL RESEARCH article

Urtica diocia L. ameliorative efficiency against CCl4-induced hepatic fibrosis in rats

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Abstract: Carbon Tetrachloride (CCl4) is a hepatotoxin causing hepatic necrosis, release of proinflammatory and profibrotic cytokines, and increased oxidative stress. *Urtica dioica* L. (stinging nettles) is traditionally used as having medicinal and pharmaceutical benefits and uses in cosmetic and food industries. Leaves are rich in bioactive compounds. Extracts of nettle leaves and roots possess antibacterial, antioxidant, anti-inflammatory, antiviral, anti-carcinogenic, and anti-rheumatic properties. This study aimed to illustrate the potential efficiency of nettle against CCl4-induced liver fibrosis in male Albino rats. Rats were divided into five groups: untreated group (**I**), an olive oil group (**II**) given 0.2 ml/100 gm olive oil by intraperitoneal injection, Nettle leaves water extract group (**III**) treated with nettle water extract (2.0 ml/kg), CCl4 group (**IV**), and CCl4-injured and nettle-treated group (**V**). After four weeks, rats were decapitated, and liver tissue and blood samples were obtained. CCl4 injection raised liver biomarkers and lipid peroxidation, decreased antioxidant levels, induced histopathological alterations in the liver, and induced DNA damage. Treatment with nettle restored the normality of the liver and overcome oxidative stress.

Introduction

Chronic liver injury induces abnormal tissue regeneration, which leads to continuous activation of fibroblasts and excessive deposition of extracellular matrix, a pathological case known as liver fibrosis [1]. Several causes including viral hepatitis, alcoholic liver, autoimmune diseases, fatty liver, and chemical exposure, are expected promoters for liver fibrosis [2]. CCl4 is a hepatotoxin causing hepatic necrosis, release of proinflammatory and profibrotic cytokines, and increased oxidative stress [3]. CCl4 toxicity is related to extensive production of free radicals and reactive oxygen species. Free radicals are unstable molecules that react with lipids, proteins, carbohydrates, and DNA, resulting in cellular membrane damage, lipid peroxidation, and DNA fragmentation ending with cell death [4]. Medicinal plants have different pharmacological activities depending upon the active compounds that they contain [5-8]. However, other plants have liver toxicity [9, 10]. *Urtica dioica L.* (stinging nettles) is traditionally used as having medicinal and pharmaceutical benefits and uses in cosmetic and food industries. This plant is gaining attention as a highly nutritious food, where fresh leaves are dried and used as powder or in other forms. Leaves are rich in many bioactive compounds. Nettles are widely distributed throughout Europe, North Africa, North America, and some parts of Asia. Extracts of nettle leaves and roots possess antibacterial, antioxidant, anti-inflammatory, antiviral, anti-carcinogenic, and anti-rheumatic efficiency [11]. These variable benefits of nettle are related to its sufficient ingredients such as terpenes, polyphenolic compounds, carotenes, vitamins, minerals, fatty acids and amino acids [12]. Thus, the aim of the study was to illustrate the potential efficiency of nettle against CCl4-induced liver fibrosis in rats.

Materials and methods

Chemicals: CCl4 was purchased from Sigma-Aldrich chemicals. It was diluted with olive oil 1: 1 ratio. The kits for measuring alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), albumin, total protein, creatinine, urea, uric acid, superoxide dismutase (SOD), catalase, and malondialdehyde (MDA) had been acquired from Bio-agnostic, Egypt.

Plant: The leaves of nettle (*Urtica dioica* L.) were purchased from a local herbalist and identified by a Botanist at the Department of Botany, Sebha University. The dried nettle leaves were ground in a machine that grinds to get a fine powder. To prepare a water extract of *Urtica dioica* L. leaves. 10 g of leaves powder was put in a thimble, soaked in Soxhlet overnight, after that extraction is achieved by using 100 ml of distilled water for 10.0 hrs. at 90.0°C. By using a rotary evaporator, the extract was collected into conical flasks separately and concentrated at low temperature and lower pressure. The dried extract is stored in the freezer until use [13].

Experimental animals: 50 adult male Albino Wistar rats (body weight of 140-150 g) were housed in plastic boxes under standardized conditions (temperature, 22.0±3.0°C and 12/12 hrs. cycle of light and dark), and they were provided a standard diet in the college's animal house. The experimental protocol was approved by the Institutional Animal Ethics Committee of the institute (0012/2024).

Experimental design: Rats were adapted for one week and received a regular diet. After adaptation time, rats were divided into five groups, 10 rats in each group, as the follows:

Group I (control rats): rats were subjected to standard conditions.
Group II (vehicle): rats were i.p. injected with 0.2 ml/100 gm twice weekly at equal intervals for four weeks.
Group III (Nettle leaves aqueous extract): rats were daily i.p. injected with 2.0 ml/kg for four weeks.
Group IV (CCl4): CCL4 was mixed equally with olive oil in a ratio. Dose of CCl4 was i.p. at two stages. First stage rats were injected with 0.2 ml/100 gm of the 1: 1 ratio CCl4: Olive oil mix, twice weekly at equal intervals for two weeks. Second-stage rats were injected with 0.1 ml/100 gm of the 1: 1 mix for another two weeks [13].
Group V (Nettle water extract plus CCl4): rats received the previous CCl4 dose and received 2.0 ml/kg of Nettle leaves water extract for four weeks.

Biochemical and tissue analysis: At the end of the experiment, rats in all groups were starved overnight and sacrificed under slight chloroform anesthesia. Blood samples were collected by cardiac puncture and placed in a serum/plasma-separating tube, then centrifuged at 3000 rpm for 15.0 min, and then the resulting serum/plasma was stored in a freezer at -20.0°C for biochemical parameters. To estimate tissue parameters (catalase, SOD and MDA), the liver of the rat was dissected out and liver homogenate was prepared in 0.1 M potassium phosphate buffer (pH 7.4). The homogenate was then centrifuged at 4.0ºC at 3000 rpm for 10.0 min, and the supernatant was used in measurements.

Histopathological analysis: Liver tissues were fixed in 10.0% formalin for at least 24 hrs., embedded in paraffin, and cut into 5.0 µm-thick sections using a rotary microtome. The sections were stained with Hematoxylin-eosin stain and Masson's Trichrome stain and observed under the microscope for any histopathological changes in the liver.

Comet assay: One gram of crushed liver samples was transferred to 1.0 ml ice-cold PBS. This suspension was stirred for five min and filtered. Cell suspension (100 ul) was mixed with 600 ul of low-melting agarose (0.8% in PBS). 100 ul of this mixture was spread on pre-coated slides. The coated slides were immersed in lysing buffer (0.045 M TBE, pH 8.4, containing 2.5% SDS) for 15.0 min. In the electrophoresis chamber containing the same TBE buffer, the slides were mounted but devoid of SDS. The conditions for electrophoresis were 2.0 V/cm for 2.0 min. Stain with ethidium bromide 20.0 ug/ml at 4.0°C. The comets tail lengths were measured from the middle of the nucleus to the end of the tail with a 40x increase for the count and measure the size of the comet. Kinetic Imaging, Ltd. created the Comet 5 image analysis software (Liverpool, UK) by measuring the length of DNA migration. A microscope linked to a CCD camera was used to assess the quantitative and

qualitative extent of DNA damage in cells and the percentage of migrated DNA. The Comet's tails extents were measured from the mid of the nucleus to the end of the tail with a 40x objective for the total and measurement of the size of the Comet [14].

DNA fragmentation: Tissues homogenization (100 mg) in hypotonic lysis buffer (0.2% Triton X-100, 10 mM TRIS, and 1.0 mM EDTA, pH 8.0), then centrifugation of cell lysate at 10,000 rpm for 15.0 min at 4.0ºC to separate intact chromatin in the pellet from fragmented/damaged DNA in the supernatant.

For gel electrophoresis: Half of the supernatant containing the separated small DNA fragments can be used for gel electrophoresis. Where an equal volume of absolute isopropanol and a tenth volume of 5.0 M NaCl were added to the supernatant to precipitate DNA, then kept overnight at -20.0ºC. The precipitation samples were centrifuged at high speed for 30.0 min, and then washed with 70.0% ethanol by centrifugation for 10.0 min. After drying at room temperature, the pellet was reconstituted with TE (10.0 mM Tris Cl, 1.0 mM EDTA, pH 7.4). To visualize the DNA fragmentation, the samples were mixed in loading dye (0.25% bromophenol blue, 0.25% xylene cyanole, and 30.0% glycerol) and electrophoresed on 1.5% agarose gel stained with ethidium bromide (0.5 µg/ml of 10. mg/ml) in 1x TBE buffer at 5.0 volts for 2.0 hrs. and visualized on a UV transilluminator. The intact DNA pellet was resuspended in the hypotonic solution. Then both the pellet fractions and the other half of the supernatant were treated with 0.5 ml of 25.0% trichloroacetic acid and were left overnight at 4.0ºC then, DNA was precipitated by centrifugation. An 80.0 µl of 5.0% trichloroacetic acid was added to the precipitated DNA of fractions and incubated at 90.0ºC for 20.0 min and centrifuged at 10000 rpm for 10 min. Then 1.0 ml of freshly prepared diphenylamine solution [1.5 g of diphenylamine dissolved in 100 ml acetic acid, 1.5 ml of sulfuric acid, and 0.50 ml of acetaldehyde (16.0 mg/ml)] was added to each sample fraction and incubated at 4.0ºC for 24.0 hrs.

Measure the optical density at 600 nm: Calculation of DNA fragmentation percent using the following equation: The proportion of fragmented DNA was calculated from the absorbance reading at 600 nm. DNA fragmentation=OD of fragmented DNA (supernatant)/[OD of fragmented DNA (supernatant) + OD of intact DNA (pellet)]×100.

Statistical analysis: The significance of the difference among groups for each tested parameter was evaluated by Analysis of Variance (one-way ANOVA) followed by post-hoc Tukey's HSD test. $P<0.05$ was considered significant.

Results

Biochemical and oxidative stress markers: Liver function biomarkers (ALT, AST, ALP, GGT, Total protein, and albumin) were estimated in different rat groups. The levels of ALT, AST, ALP, GGT, albumin, and total protein were not significantly different between control, olive oil, and nettle groups. In the CCl4 group, ALT, AST, ALP, and GGT levels were highly significantly increased, and levels of total protein and albumin were highly significantly decreased compared to the control group. While in the nettle and CCl4 group, ALT, AST, ALP, and GGT levels were highly significantly decreased, and levels of total protein and albumin were highly significantly increased compared to the control (**Figures 1-6**).

The level of MDA was significant difference in the level of MDA between the control, olive oil, and nettle groups. Its level in the CCl4 group was highly significantly increased. Compared to the CCl4 group, the level of MDA was significantly decreased in the nettle and CCl4 group (**Figure 7**). To estimate the antioxidant activity, the levels of SOD and CAT were measured in rat groups. Hepatic levels of SOD and CAT were not significantly different between control, olive oil, and nettle groups. Their levels were significantly decreased in the CCl4 group compared to the control. In the nettle and CCl4 group, the levels of SOD and CAT were highly significantly increased when compared with the CCl4 group (**Figures 8** and **9**).

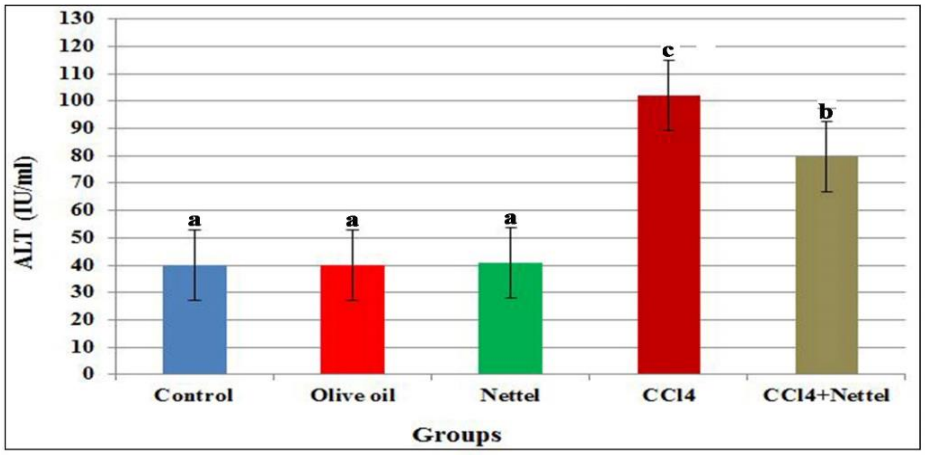

Figure 1: Alanine aminotransferase level in different rat groups

Standard errors are indicated on the bars. There is no significant difference between the values with the same letter
Values with different letters are significantly different

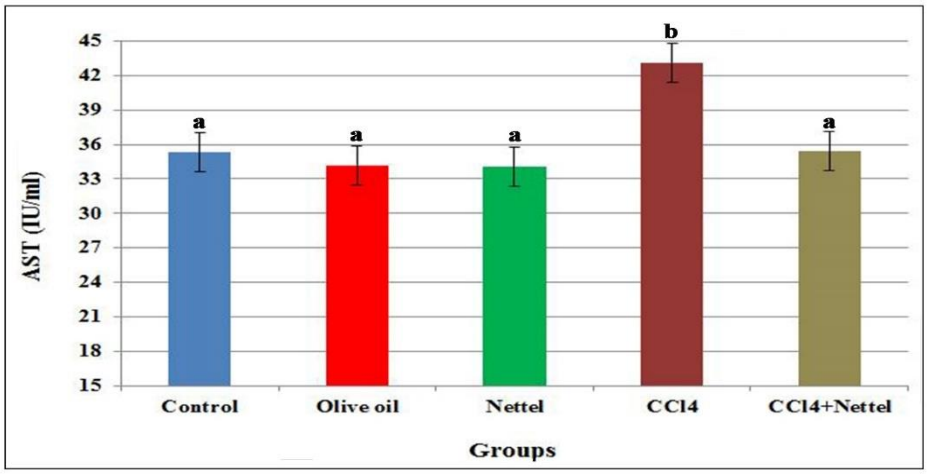

Figure 2: Aspartate amino transferase level in different rat groups

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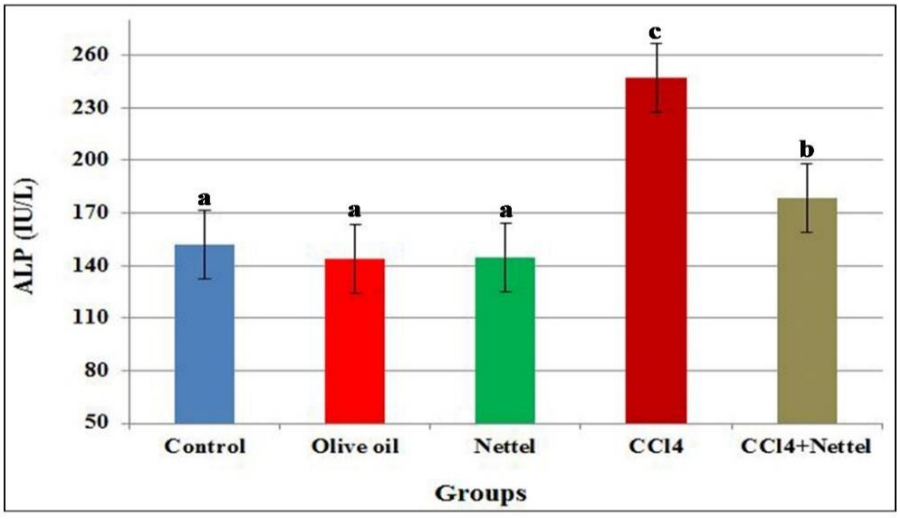

Figure 3: Alkaline phosphatase level in different rat groups

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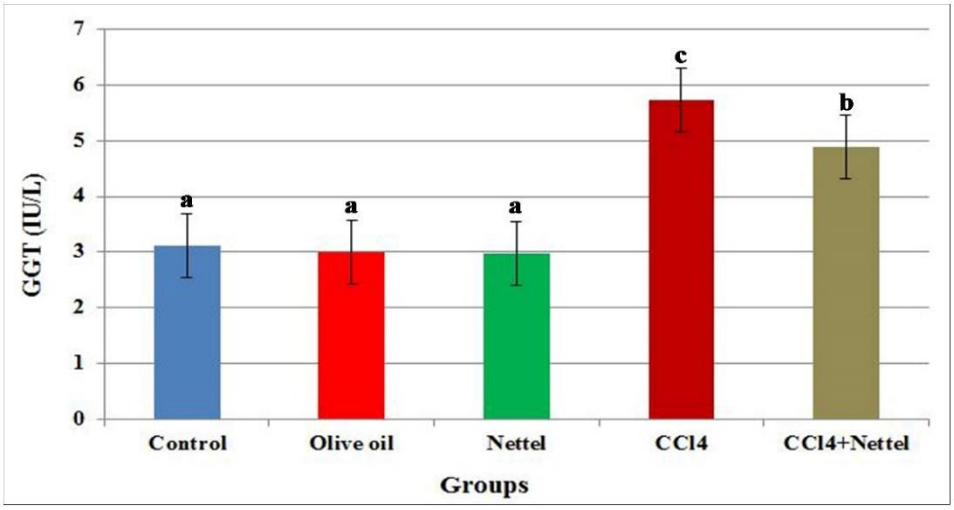

Figure 4: Gamma-glutamyl transferase level in different rat groups

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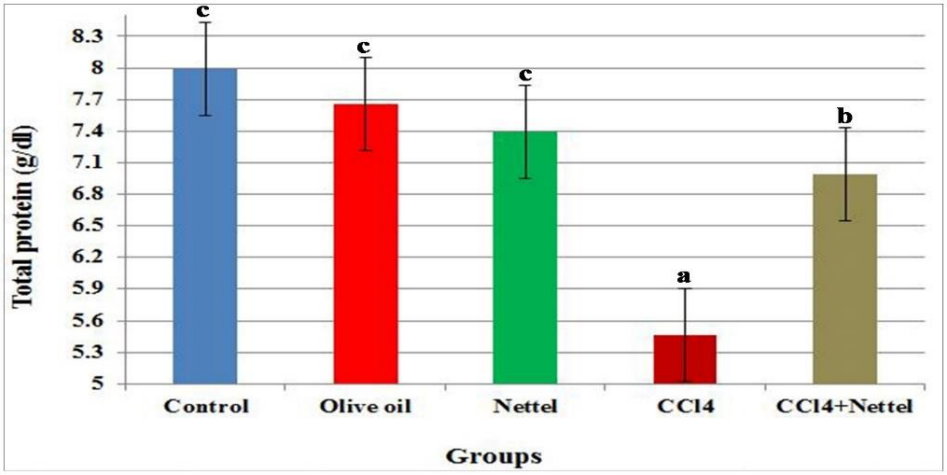

Figure 5: Total protein level in different rat groups.

Standard errors are indicated on the bars. There is no significant difference between the values with the same letter
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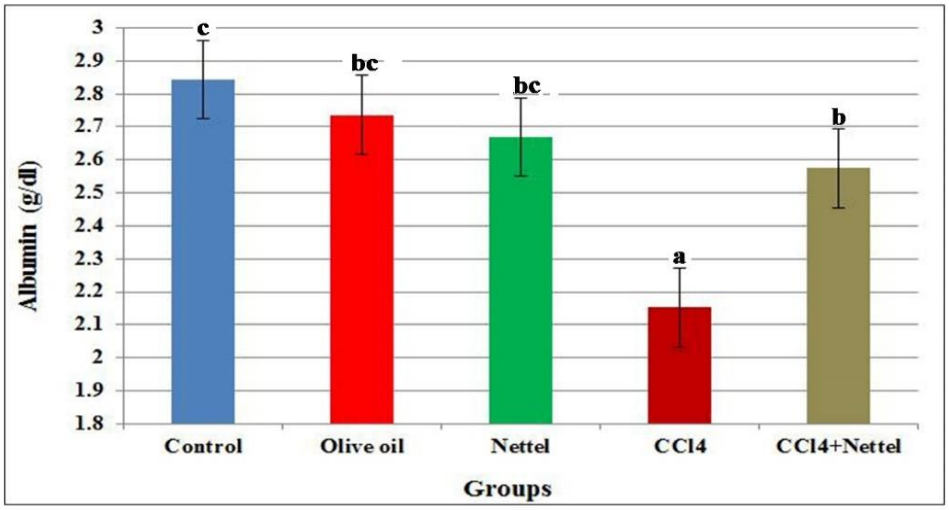

Figure 6: Albumin level in different rat groups.

Standard errors are indicated on the bars. There is no significant difference between the values with the same letter
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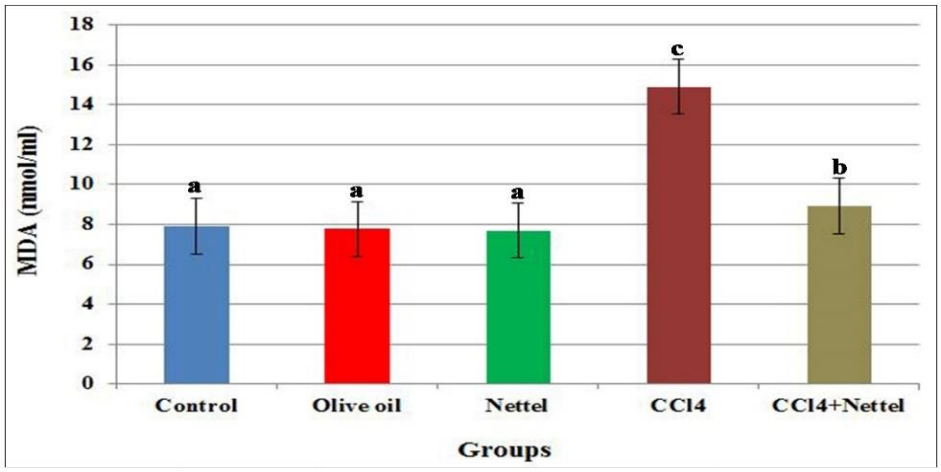

Figure 7: Malondialdehyde level in different rat groups

Standard errors are indicated on the bars. There is no significant difference between the values with the same letter
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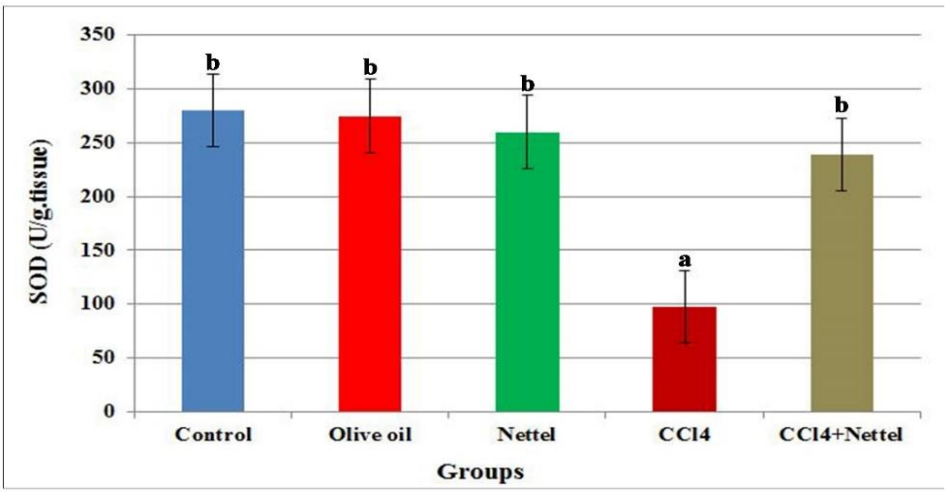

Figure 8: Superoxide dismutase level in different rat groups

Standard errors are indicated on the bars. There is no significant difference between the values with the same letter
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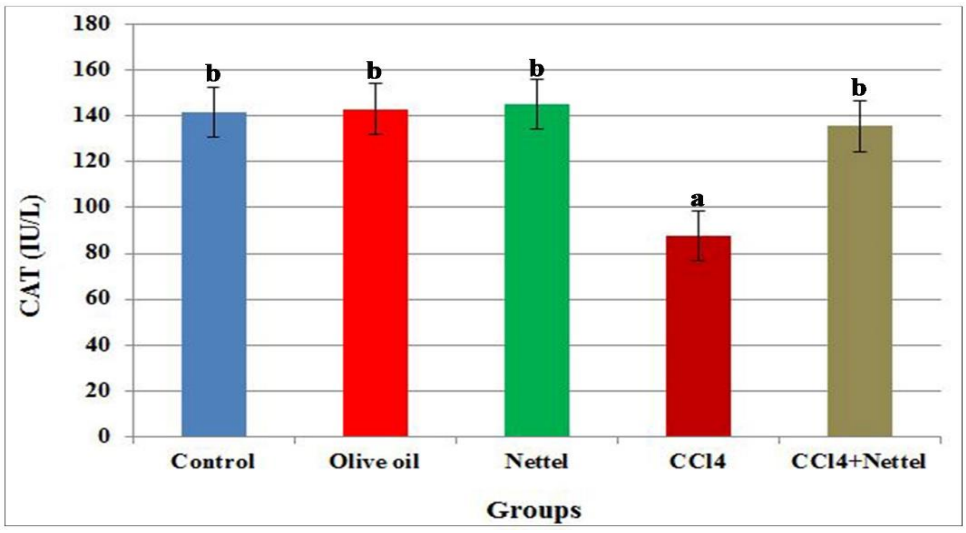

Figure 9: Catalase levels in different rat groups

Standard errors are indicated on the bars. There is no significant difference between the values with the same letter
Values with different letters are significantly different

Histopathological examination

H & E stain: Microscopic examination of H & E-stained liver tissues of control, olive oil, and nettle groups revealed normal hepatic architecture (**Figures 10 A, B,** and **C**). Examination of H & E-stained liver tissues of the CCl4 group showed congestion of the central vein, infiltration of inflammatory cells, vacuolation of hepatocytes, pyknosis of nuclei, necrotic hepatocytes, and collagen fiber deposition in and around the central vein (**Figures 10 D** and **E**). Treating CCl4-injured rats with nettle revealed restoration of normal hepatic structure with slight congestion in blood sinusoids and inflammatory cells infiltration (**Figure 10 F**).

Masson's Trichrome stain: Microscopic examination of Masson's Trichrome-stained liver tissues of control, olive oil, and nettle groups revealed normal hepatic architecture with normal collagen deposition (**Figures 11 A, B**, and **C**). Examination of Masson's Trichrome-stained liver tissues of the CCl4 group showed abnormal hepatic architecture with severe deposition of collagen fibers, congestion of the central vein and necrotic hepatocytes (**Figure 11 D** and **E**). Treating CCl4-injured rats with nettle revealed restoration of normal hepatic structure with slight deposition of collagen fibers in the central vein (**Figure 11 F**).

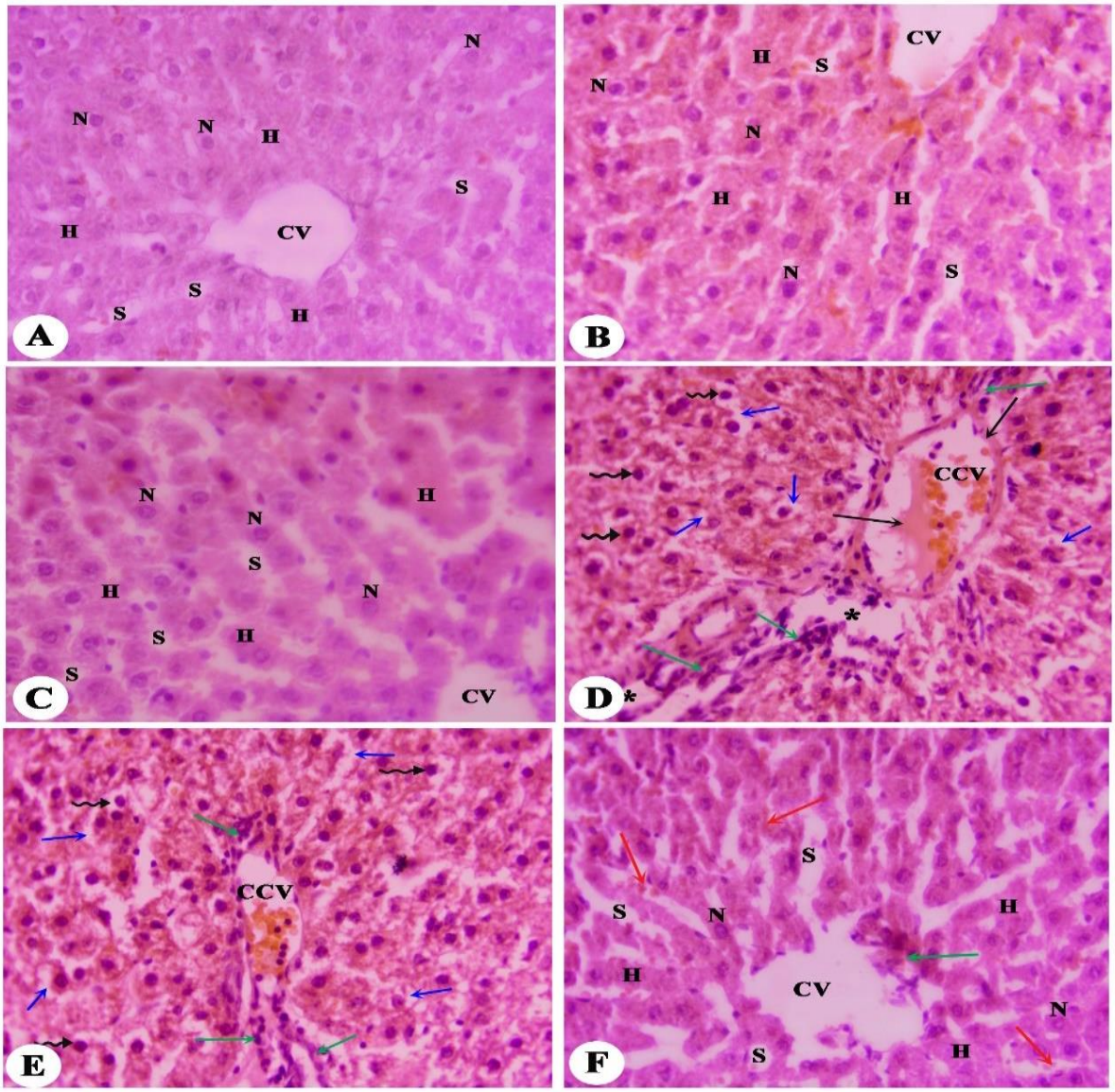

Figure 10: Liver tissue sections of different rat groups stained by H&E.

Control (A), olive oil (B), and Nettle (C) groups showed normal structure. CCl4 (D and E) group showed deposition of collagen fibers (black arrow), vacuolation of hepatocytes (blue arrow), pyknotic nuclei (twisted arrow), inflammatory cell infiltration (green arrow), necrosis of hepatic cells (*), and congested blood vessel (CCV). Nettle treated (F) group showed restored structure with congested sinusoids (red arrow) and inflammatory cell infiltration (green arrow). N: nuclei, S: blood sinusoid, H: hepatocytes, and CV: central vein.

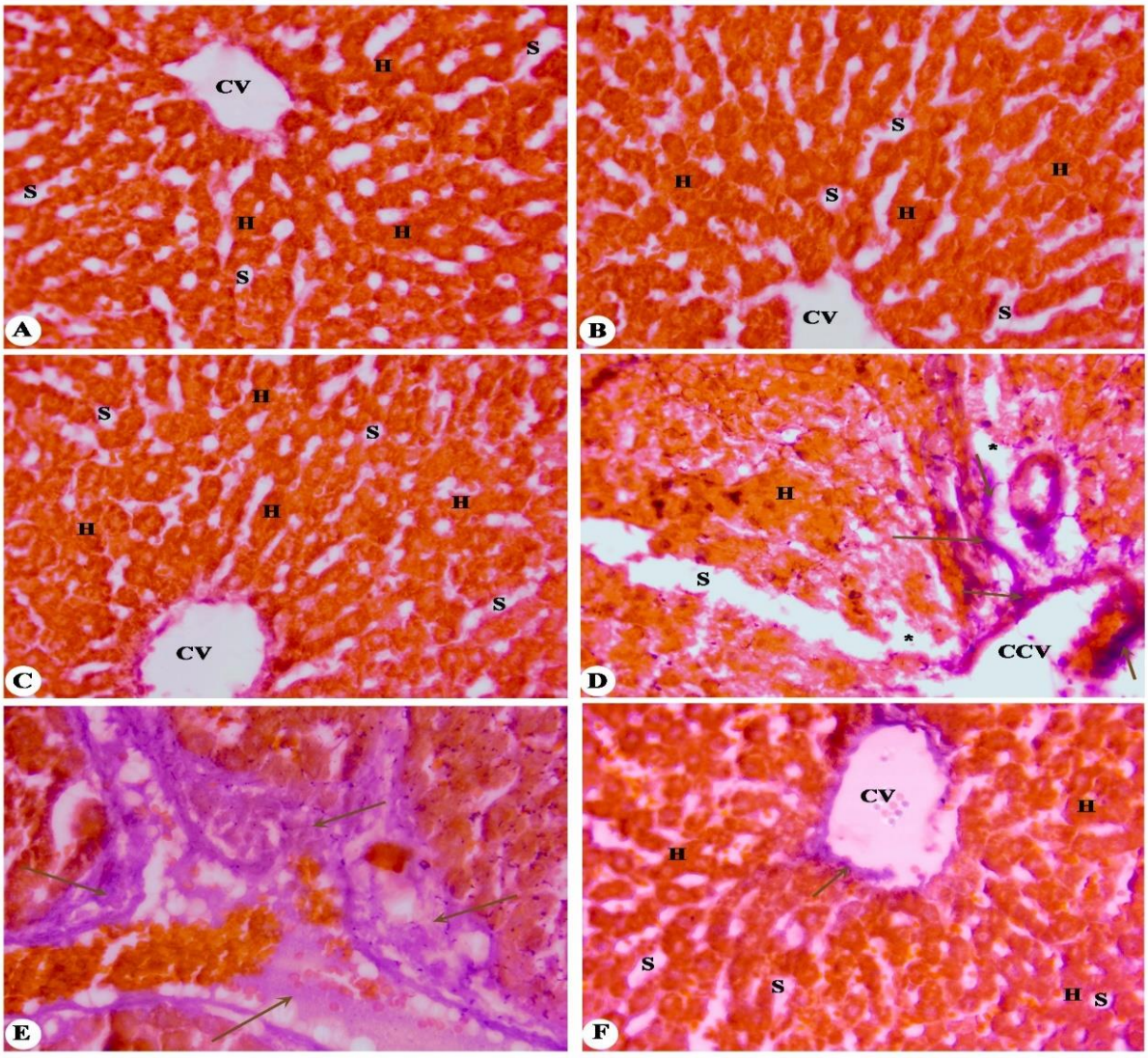

Figure 11: Liver tissue sections of different rat groups stained by Masson's Trichrome

Control (A), olive oil (B), and Nettle (C) groups showed normal structure with normal collagen deposition. CCl4 (D and E) group showed severe deposition of collagen fibers (arrow), necrosis of hepatic cells (*), and congested blood vessel (CCV). Nettle treated (F) group showed restored structure with slight collagen deposition (arrow). N: nuclei, S: blood sinusoid, H: hepatocytes, and CV: central vein.

Comet assay: Comet tail DNA percent in rats treated with CCl4 was significantly increased compared to the control group. While nettle significantly reduced the effect of CCl4 on the liver cells (**Table 1** and **Figure 12**) when compared to the control group (10.033). Nettle and olive oil had no significant effect on tail DNA percent (17.4333 and 17.5333, respectively). There was a sharp increase in tail DNA percent in the CCL4 group (25.4333), indicating the genotoxicity of CCL4. This increase in tail DNA percent of CCl4 was decreased after being treated with nettle to (11.7333), which implies the protection ability of nettle.

Table 1: Rates of Comet tail DNA percent in the liver of different rat groups

Group	Percent tailed	Tail moment
Control	10.03333	0.5047
Olive oil	17.53333	0.88922
Nettle	17.43333	0.68169
CCl4	25.43333**	1.07138
CCl4+nettle	11.73333**	0.81403

**P<0.01

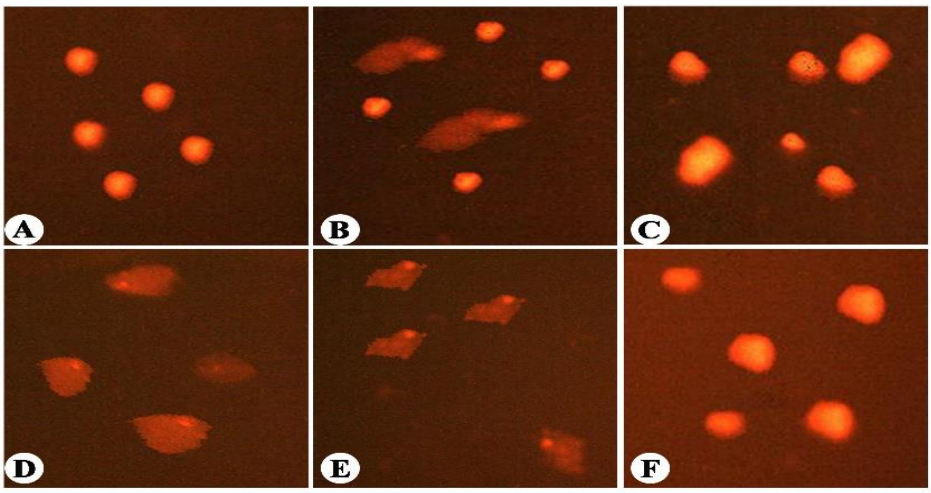

Figure 12: Comet photomicrographs showing the DNA migration pattern in hepatocytes of rats

A: Control, B: Olive oil, C: Nettle, D and E: CCl4, and F: CCl4 plus nettle

DNA fragmentation: The results of percent DNA fragmentation indicated that the percentage of DNA fragmentation is significantly increased in the group treated with CCl4 compared to the control group. However, rats treated with CCl4 and given nettle showed a significant improvement in the percentage of DNA fragmentation compared to the control values.

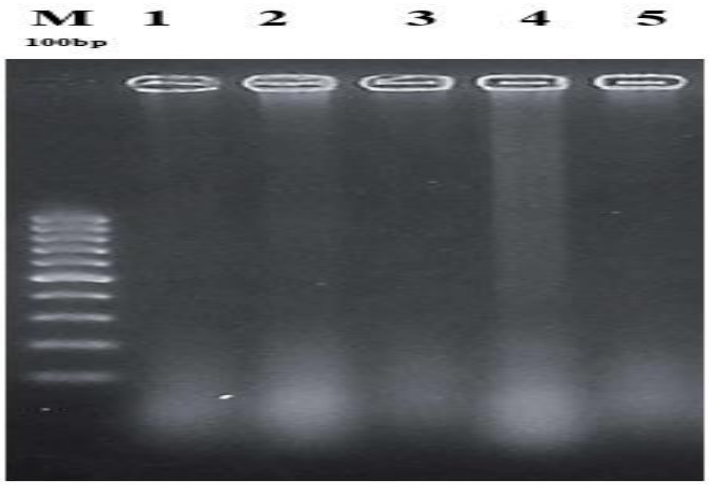

Figure 13: Analysis of liver tissue DNA fragmentation on 1.5% agarose gel electrophoresis in 1x TBE buffer

Lane 1: Control, Lane 2: Olive oil, Lane 3: Nettle, Lane 4: CCL4 and Lane 5: CCl4 plus nettle

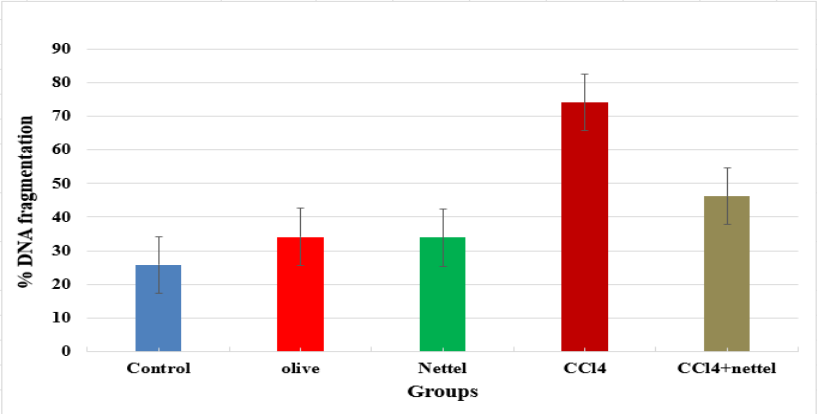

Figure 14: DNA fragmentation percentage in different rat groups

Standard errors are indicated on the bars

Discussion

Free radicals resulting from the metabolism of many exogenous toxic chemicals inside the hepatic tissue may be more toxic than the parent compound [15]. CCl4 is metabolized by cytochrome P4502E1 to trichloromethyl radical (CCl3*). In the presence of oxygen, CCl3* radical is converted to the trichloromethyl-peroxy radical (Cl3COO*). These radicals dramatically change the properties of hepatic biological membranes [16], causing leakage of cytosolic enzymes into the blood circulation [17]. The present results revealed that exposure of rats to CCl_4 caused significant increase in the levels of ALT, AST, ALP, and GGT and significant decrease in the levels of total protein and albumin. The results are in harmony with the previous findings [15, 18]. Free radicals produced from the metabolism of CCl4 cause cell destruction and elevate lipid peroxidation. CCl4 causes oxidative stress in hepatic tissue by disrupting the balance between oxidative and antioxidant systems, resulting in increased free radical generation and reduced antioxidant defenses [19]. CCl4 suppresses the functionality of key antioxidant enzymes, including CAT, SOD, GSH-Px, and GST [20-22]. In addition, CCl4 elevates MDA, which is a known indicator of oxidative stress [23, 24]. Results of the current study are inconsistent with these previous findings and revealed a significant increase in MDA level and a significant decrease in SOD and CAT levels in rats treated with CCL4.

In the current study, administration of CCL4 resulted in loss of hepatic architecture and severe liver tissue injury, including degeneration and necrosis of hepatocytes in the form of large vacuoles in the cytoplasm and small pyknotic nuclei. In addition to congestion of blood vessels, dilatation of sinusoids, an inflammatory infiltrate, and finally fibrosis. In agreement with the previous findings that demonstrate CCL4 injection induced liver injury [25, 26]. Hepatocytes showed necrosis, vacuolations, inflammation, and fibrosis. Masson's Trichome stained sections of rats of CCl4 revealed increased collagen fibers deposition. These findings are in agreement with the previously published data [27, 28] that revealed increased collagen deposition in Masson's-stained sections of livers of CCL4 treated rats. CCL4 causes oxidative stress that result in HSCs activation which change to myofibroblasts and become the main source of collagen and cytokine secretion [29]. Alkaline comet assay detects single/double strand breaks, alkali-labile sites (resulting from DNA base losses), DNA cross-links, and apoptotic nuclei. These DNA errors may be repaired, may be lethal to the cells, or may be fixed into a mutation [30]. In the current study, the exposure of CCl4 increased the DNA breakage (tail moment, percent DNA in the tail) in the rat liver. Going with these findings, a previous study revealed drastically increased DNA strand breakage (tail moment, percent DNA in the tail) in rat blood lymphocytes [31]. CCl4 is well known to degrade DNA in liver tissue of rats by generating free radicals and increasing DNA fragmentation [15]. CCl4 treatment led to significant DNA fragmentation and the production of a DNA smear on an agarose gel, which suggests CCl4-induced hepatic injury [32]. Currently, liver damage induced by CCl4 was confirmed by DNA fragmentation percentage and DNA ladder assay banding pattern, and revealed cellular correlates of damage after CCl4 administration. *Urtica dioica* L. includes compounds such as steroids, terpenoids, phenylpropanoids, lignans, coumarins, polysaccharides, lectins, and flavonols [33]. The phenolic compounds of *Urtica dioica* L. have antioxidative action. They stabilize lipid peroxidation. Polyphenolic compounds in *Urtica dioica* L. may have inhibitory effects on mutagenesis and carcinogenesis [34, 35].

In this study, treating CCl4-injured rats with nettle revealed a significant decrease in ALT, AST, ALP, and GGT activities, and the total protein and albumin were significantly increased approving its protective efficiency. Administration of nettle to CCl4-injured rats results in a significant decrease in MDA and a significant increase in SOD and CAT. These are in agreement with the previous studies [36-38], which are related to *Urtica dioica* L. strong hydrogen donating ability, a metal chelating ability, and effectiveness as scavengers of hydrogen peroxide, the superoxide anion, and free radicals [39]. The oxidative damage can eventually lead to DNA mutagenesis and cell death. *Urtica dioica* L. has an antioxidant activity so its administration may be effective against DNA fragmentation. At present, CCl4-injured rats treated with *Urtica*

dioica L. showed a decline in the percentage of DNA fragmentation. *Urtica dioica* L. hepatoprotective abilities may be a result of the presence of flavonoids, polyphenols, ascorbic acid, carotenoids, tannins, and lignins among the plant constituents. They are free radical scavengers and hence promote hepatoprotection [40]. Histological analysis revealed restoration of the normal hepatic architecture with congestion of some blood sinusoids and slight infiltration of inflammatory cells, and slight deposition of collagen fibers.

Conclusion: The findings indicate that *Urtica dioica* L. decreases lipid peroxidation and liver enzymes, and increase antioxidant defense system activity in the CCl4-treated rats.

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